Defining Key Signaling Nodes and Therapeutic Biomarkers in NF1-Mutant Cancers

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ABSTRACT

NF1 encodes a RAS GTPase-activating protein. Accordingly, aberrant RAS activation underlies the pathogenesis of NF1-mutant cancers. Nevertheless, it is unclear which RAS pathway components represent optimal therapeutic targets. Here, we identify mTORC1 as the key PI3K effector in NF1-mutant nervous system malignancies and conversely show that mTORC2 and AKT are dispensable. However, we find that tumor regression requires sustained inhibition of both mTORC1 and MEK. Transcriptional profiling studies were therefore used to establish a signature of effective mTORC1–MEK inhibition in vivo. We unexpectedly found that the glucose transporter GLUT1 was potently suppressed, but only when both pathways were inhibited. Moreover, unlike VHL- and LKB1-mutant cancers, reduction of 18F-FDG uptake required the suppression of both mTORC1 and MEK. Together, these studies identify optimal and suboptimal therapeutic targets in NF1-mutant malignancies and define a noninvasive means of measuring combined mTORC1–MEK inhibition in vivo, which can be readily incorporated into clinical trials.

SIGNIFICANCE: This work demonstrates that mTORC1 and MEK are key therapeutic targets in NF1-mutant cancers and establishes a noninvasive biomarker of effective, combined target inhibition that can be evaluated in clinical trials. Cancer Discov; 4(9); 1062–73. ©2014 AACR.
INTRODUCTION

The NF1 tumor suppressor is mutated or suppressed in a variety of sporadic cancers, including glioblastoma, neuroblastoma, melanoma, and non-small cell lung cancer (1–5). NF1 mutations also underlie the familial cancer syndrome neurofibromatosis type 1 (NF1; refs. 6 and 7). Patients with NF1 exhibit a variety of tumorigenic and nontumorigenic manifestations, but the most common cause of death is malignant peripheral nerve sheath tumors (MPNST). These highly aggressive tumors are lethal in approximately 70% of patients, and conventional chemotherapy and radiation do not reduce mortality in individuals with inoperable tumors (8–10). Therefore, developing effective targeted therapies for these individuals represents an important and unmet clinical need. Moreover, an effective therapy for this tumor type may be more broadly applicable to other sporadic NF1-mutant cancers.

The NF1 tumor suppressor gene encodes a RAS GTPase-activating protein (GAP), which inactivates RAS by catalyzing the hydrolysis of RAS-GTP (6, 7). As such, when NF1 is mutated or suppressed, RAS and downstream effectors become hyperactivated (11). Both the PI3K–mTOR and MEK–ERK pathways have been shown to be important in various NF1-mutant tumors, and therefore components of these pathways represent potential therapeutic targets (12–15). However, given the plethora of available drugs that target these pathways, we set out to genetically and chemically deconstruct the most important signaling nodes in NF1-mutant MPNSTs. Together with preclinical studies in a genetically engineered mouse tumor model, we found that mTOR complex 1 (mTORC1) is the key PI3K pathway component in these NF1-mutant malignancies, AKT and mTORC2 are dispensable, and only sustained mTORC1 and MEK inhibition promotes tumor regression.

Several combined PI3K–MEK pathway trials are in development or are being considered for other cancers (16). However, the clinical challenge will be to identify a drug combination and dose that effectively suppresses both pathways, while minimizing toxicity. It is currently unclear how dosing can be adjusted while confirming that both targets are sufficiently inhibited in real time, especially given that the duration of inhibition seems to be an important determinant of efficacy. Thus, establishing a tractable biomarker for effective, combined target inhibition would greatly facilitate this effort. By performing transcriptional profiling and imaging studies, we unexpectedly identified GLUT1, which mediates [18F]-fluorodeoxyglucose ([18F]-FDG) uptake, as a key gene that is suppressed before tumor regression but only when both pathways are effectively inhibited. Moreover, we show that [18F]-FDG uptake is a reliable readout of combined target inhibition. This insight can be directly applied to the design of clinical trials in NF1-mutant cancers and may also have broader utility in other RAS-driven tumors.
RESULTS

p110α and mTORC1 Are the Key Effectors in NF1-Mutant Nervous System Malignancies

We have previously shown that loss or inactivation of NF1 triggers the aberrant activation of PI3K-mTORC1 signaling in human and mouse MPNSTs (17). However, it is currently unclear which specific components within this pathway represent the best therapeutic targets. Such insight would reveal which drugs should be preferentially evaluated or excluded in clinical trials. Therefore, we sought to genetically and chemically deconstruct this pathway in NF1-mutant MPNSTs. There are three class 1A catalytic PI3K isoforms: p110α, p110β, and p110δ. Although p110α is frequently mutated in human cancer, p110β has been shown to play an essential role in PTEN-mutant cancers, and p110δ is frequently mutated in human cancer. p110β is critical in chronic lymphocytic leukemia (18–20). To identify which catalytic isoforms are essential in NF1-mutant nervous system malignancies, we first assessed the biologic effects of isoform-specific ablation in human MPNST cells derived from patients with NF1. Although all three isoforms were present in MPNSTs, genetic ablation of p110α, but not p110β or p110δ, dramatically impaired the proliferation of both tumor lines (Fig. 1A). Similarly, NF1-mutant glioblastoma (GBM) cells were exclusively sensitive to siRNA-mediated depletion of p110α, but not p110β or p110δ, suggesting that p110α may play a more general role in NF1-deficient cancers (Fig. 1A). To complement these findings, we used PI3K isoform–specific inhibitors: the p110α-specific inhibitor A66-(S), the p110β-specific inhibitor AZD-6284, and the p110δ-specific inhibitor CAL-101, as well as GDC-0941, a pan-PI3K inhibitor (21–24). The reported specificities of each drug are outlined in Supplementary Table S1. In human MPNST cell lines, the p110α-specific inhibitor A66-(S) and GDC-0941 potently inhibited the phosphorylation of AKT and S6; however, the p110β- or p110δ-specific inhibitors, AZD-6284 and CAL-101, respectively, did not suppress the phosphorylation of either protein (Fig. 1B).

mTOR functions in two distinct complexes: the rapamycin-sensitive complex mTORC1, which phosphorylates 4E-BP1...
and S6 kinase, and the relatively rapamycin-insensitive complex mTORC2, which phosphorylates AKT at serine 473 (25, 26). NF1-deficient MPNSTs have been shown to be sensitive to rapamycin, indicating that mTORC1 plays a role in this tumor type; however, the contribution of mTORC2 activity, if any, to MPNST growth is unknown (13, 17). We genetically targeted essential component proteins of each complex to evaluate the relative contribution of these two complexes. RAPTOR is an essential component of mTORC1, but is not present in mTORC2, whereas RICTOR, a primary component protein of mTORC2, is not a member of the mTORC1 complex (27, 28). As expected, siRNA-mediated loss of RAPTOR or mTOR suppressed S6 phosphorylation and led to impaired proliferation of MPNST cell lines (Fig. 1D). However, loss of RICTOR had no effect on MPNST proliferation, despite the effective suppression of phosphorylation of the mTORC2 target AKT (Fig. 1D).

To further evaluate a role, or lack thereof, for AKT, tumors were treated with the allosteric AKT inhibitor MK-2206 (29). MK-2206 suppressed the phosphorylation of AKT at S473 and T308 and effectively inhibited AKT kinase activity, as confirmed by the loss of TSC2 phosphorylation on T1462 (Fig. 1E and Supplementary Fig. S1). However, unlike rapamycin, MK-2206 had no effect on the proliferation of NF1-mutant MPNST cells (Fig. 1E). The mTOR kinase inhibitor Torin1 inhibits both the mTORC1 and mTORC2 complexes. Notably, Torin1 has been reported to more effectively inhibit mTORC1, as compared with rapamycin, and in particular more potently suppresses 4E-BP1 phosphorylation, as observed in these studies (Fig. 1E and Supplementary Fig. S1). Accordingly, Torin1 potently suppressed the proliferation of NF1-mutant cells and did so better than rapamycin (P < 0.02). As noted, both MK-2206 and Torin1 equivalently and potently suppressed AKT phosphorylation and activity, although only Torin1 suppressed MPNST cell proliferation. Moreover, MK-2206 did not enhance the antiproliferative effects of rapamycin (Fig. 1F). Taken together, these results suggest that mTORC1 is a critical effector in NF1-mutant cancers and that mTORC2 and AKT are dispensable in these tumor cells.

Selection of an Effective PI3K–mTOR Pathway Inhibitor

These in vitro studies suggested that pan-PI3K inhibitors, p110α-specific inhibitors, or mTORC1 inhibitors should suppress the growth of NF1-mutant MPNSTs. Therefore, we first...
evaluated the in vivo effects of GDC-0941 and rapamycin on a genetically engineered mouse MPNST model. Like human MPNSTs, tumors from these animals harbor compound mutations in \( \text{Nf1} \) and \( \text{Trp53} \) and develop with an average latency of 5 months. These MPNSTs are highly aggressive, and mice survive for an average of 10.7 days after tumors are detected, thus recapitulating the aggressive nature of human tumors (30). As previously shown, rapamycin suppressed the growth of \( \text{Nf1}/\text{Trp53} \)-mutant MPNSTs (\( P < 0.0001; \) ref. 13); however, GDC-0941 did so significantly less well (\( P = 0.0021; \) Fig. 2A). Notably, the MTD of GDC-0941 (150 mg/kg) inhibited the phosphorylation of AKT, S6, and 4E-BP1 in tumors within 1 hour; however, these pathways were reactivated within 4 hours after treatment (Fig. 2B). In contrast, rapamycin suppressed S6 and 4E-BP1 phosphorylation for at least 18 hours, consistent with the observed enhanced efficacy and the demonstrated importance of mTORC1 in these tumors. It should be noted that AKT is not activated by relief of feedback mechanisms in this model, as we have previously shown (Fig. 2B; ref. 13, 31). Several other PI3K–mTOR pathway inhibitors, including BEZ-235, Torin2, and INK-128, were evaluated in these animals (data not shown); however,
we were unable to identify an inhibitor that exhibited better pharmacodynamics or growth inhibition than rapamycin at tolerable doses in these animals. Therefore, rapamycin was selected for further studies.

**Combined, Sustained Inhibition of mTORC1 and MEK Promotes MPNST Regression In Vivo**

Although mTORC1 is a critical signaling node in NF1-mutant tumors, mTORC1 inhibition exerted only cytostatic effects on MPNSTs in vitro and in vivo (Figs. 1D and E and 2A; ref. 13). Therefore, we evaluated the effects of rapamycin combined with a MEK inhibitor, which targets a second critical RAS-effector pathway. Tumor-bearing mice were treated with vehicle, the MEK inhibitor PD-0325901, rapamycin, or the combination of rapamycin and PD-0325901. As a monotherapy, PD-0325901 slightly attenuated the growth of MPNSTs, but did so less than rapamycin (Fig. 2C). However, combined PD-0325901 and rapamycin induced tumor regression in these mice (Fig. 2C). Interestingly, these observations differ from effects observed in benign NF1-deficient peripheral nervous system tumors and myeloid malignancies, where MEK seems to function as the dominant RAS-effector pathway and MEK inhibitors exert cytotoxic effects alone, suggesting that different tumor types harboring the same initial driving genetic lesion may rely on different downstream signals (32, 33). Nevertheless, upon examining the pharmacodynamics of PD-0325901 at this dose, we found that ERK phosphorylation was inhibited for only 4 to 6 hours, whereas sustained inhibition could be achieved by dosing with PD-0325901 twice daily (Fig. 2D). As such, we hypothesized that a revised dosing schedule might exert more potent therapeutic effects. Twice-daily PD-0325901 treatment did not promote tumor regression as a monotherapy; however, when combined with rapamycin, twice-daily PD-0325901 treatment improved the therapeutic response (Fig. 2E). All mice treated with this combination responded, and more than half of the tumors regressed 50% or more, with several shrinking 75% or more. Together, these observations indicate that the duration of both MEK and mTORC1 inhibition is a critical determinant of the therapeutic response.

**Identifying GLUT1 as a Component of the Therapeutic Signature That Is Suppressed before Tumor Regression**

Pharmacodynamic markers in tumors are often not examined during clinical trials, and when they are, the kinetics of suppression are difficult to evaluate. Therefore, if a treatment does not show efficacy, especially in cases of dose escalation, it is often unclear whether the target or targets were sufficiently inhibited. Therefore, we sought to identify a molecular change that might serve as a functional biomarker of effective, combined inhibition of mTORC1 and MEK pathways. The transcriptional profiles of tumors from animals treated with vehicle, rapamycin, PD-0325901 (twice daily), or the combination of rapamycin and PD-0325901 were evaluated. Importantly, tissues were collected after 14 hours of treatment: a time point that would capture transcriptional changes caused by sustained target inhibition but occurring before tumor regression. Using a gene expression class comparison, we identified a gene set that was exclusively regulated by combined rapamycin and PD-0325901 treatment (Fig. 3A). Interestingly, Sck2a1, which encodes a glucose transporter and is commonly referred to as Glut1, was identified as one of the uniquely suppressed genes in rapamycin–PD-0325901–treated tumors (Fig. 3A). Effective mTORC1 and MEK target inhibition in tumor tissue was verified (Fig. 3B). qPCR analysis confirmed that Glut1 levels were reduced 64% after only 14 hours of treatment compared with vehicle-treated tumors and that neither rapamycin nor PD-0325901 exerted suppressive effects alone (Fig. 3C). A dramatic decrease in GLUT1 protein levels was further confirmed by evaluating its expression in tumor biopsies taken before and 3 days after treatment (Fig. 3D). These findings differ from observations in VHL- and LKB1-mutant tumors, where GLUT1 mRNA and consequently protein expression are primarily regulated by mTOR and HIF1α, and its expression can be suppressed by mTORC1 inhibitors alone (34, 35). However, in these NF1-mutant MPNSTs, suppression of both mTORC1 and the MEK–ERK pathways is required. This finding resolves a long-standing observation that rapamycin is not sufficient to suppress the expression of GLUT1 or other HIF1α target genes in vitro or in vivo in this tumor type (13). Together, these results demonstrate that GLUT1 is suppressed in MPNSTs only after combined mTORC1 and MEK inhibition, which could be exploited for developing an imaging biomarker of combined target inhibition.

**Only Combined, Effective Suppression of mTORC1 and MEK Inhibits 18F-FDG Uptake**

GLUT1 is a membrane-bound glucose transporter that is frequently overexpressed in tumors, in part because altered tumor metabolism requires increased glucose uptake (36–39). This metabolic activity can be measured by PET scans designed to quantify 18F-FDG uptake (40). GLUT1 has been shown to regulate 18F-FDG uptake in a variety of tumor types (41, 42). MPNSTs are generally FDG–PET positive, and enhanced 18F-FDG uptake is used to diagnose a conversion to malignancy, as MPNSTs often arise from benign precursor lesions (43). Because human MPNSTs exhibit a strong FDG–PET signal, and because GLUT1 was specifically suppressed in tumors treated with combined rapamycin and PD-0325901, we hypothesized that the substantial reduction in GLUT1 mRNA and protein might inhibit 18F-FDG uptake in these tumors. To evaluate this possibility, FDG–PET imaging was performed on tumor-bearing mice. As expected, MPNSTs were FDG–PET positive at baseline, mirroring the behavior of human MPNSTs (Fig. 4A). Mice were then treated with vehicle, PD-0325901, rapamycin, or PD-0325901–rapamycin, and PET analysis was performed a second time, 40 hours after the baseline scan. This time point was selected because it represents a time before detectable regression occurs, to avoid any confounding change in the FDG–PET signal due to a reduction in tumor size. It should be noted that the initial (64%) decrease in Glut1 mRNA levels can be detected 14 hours after treatment; however, given the dramatic decrease in GLUT1 protein after 72 hours, this repression is sustained and perhaps enhanced. Animals treated with vehicle, PD-0325901, or rapamycin did not have a significant change in 18F-FDG uptake after treatment (Fig. 4A and B); however, animals...
treated with both PD-0325901 and rapamycin exhibited a significant decrease in the highest standardized uptake value (SUV\textsubscript{max}; \(P < 0.004\); Fig. 4A and B). Importantly, although hexokinase and other GLUT genes can regulate glucose uptake in some settings (41, 44–46), rapamycin–PD-0325901 treatment did not affect the expression of any of these genes, suggesting that GLUT1 may be the rate-limiting step for FDG–PET uptake in MPNSTs (Supplementary Table S2).

These observations suggested that FDG–PET imaging could be used as a biomarker of effective combined MEK–mTORC1 inhibition. Such a biomarker would be invaluable in the course of evaluating similar therapies in the clinic and in the course of dose de-escalation/escalation studies. This biomarker would be particularly useful if the early changes in FDG–PET imaging were predictive of a later change in tumor size. To experimentally evaluate this possibility, we performed a dose de-escalation study in mice. Mice were treated with rapamycin in combination with 100%, 50%, or 25% of the PD-0325901 dose. As expected, this produced a range of responses in FDG–PET uptake at 40 hours and tumor regression after 10 days (Fig. 4C and D). Importantly, the suppression of FDG–PET activity at 40 hours, as measured by change in SUV\textsubscript{max}, correlated with the ultimate decrease in tumor size after 10 days (Pearson \(r = 0.711\); \(P = 0.03\); Fig. 4D and E). These results suggest that early changes in the FDG–PET signal are indicative of the degree of target inhibition and correlate with eventual tumor regression in MPNSTs treated with combined mTORC1–MEK inhibitors.

**DISCUSSION**

Numerous PI3K pathway inhibitors have been developed and are being evaluated in clinical trials (16). However, in many cancers, it is not clear which specific components within this pathway are most critical or to what degree they...
must be inhibited. Such information would undoubtedly facilitate the selection of the most appropriate drugs for clinical studies. In this study, we used a genetic and chemical approach to systematically deconstruct the PI3K signaling pathway in NFI-mutant nervous system malignancies. Importantly, we found that mTORC1, which is regulated by p110α in these tumors, is the minimal, essential PI3K pathway component, and that surprisingly AKT and mTORC2 are dispensable. However, although agents that inhibit mTORC1 promote cytostasis in human tumor cells and genetically engineered models, tumor regression requires concomitant suppression of the MEK–ERK pathway.

Notably, there are currently no effective therapies for MPNSTs. As such, these studies reveal a promising therapeutic
approach as well as a mechanistic framework for selecting the most appropriate agents for clinical trials. For example, because p110α does not seem to contribute to the therapeutic response in these tumors, perhaps p110α-specific, β-sparing PI3K inhibitors could be used with less toxicity (47). Alternatively, because mTORC1 seems to be the key PI3K effector in these tumors, perhaps rapalogues, which exhibit excellent pharmacokinetic properties, may be suitable for combination therapies. The observation that AKT is not activated in these tumors by feedback inhibition, and that AKT inhibitors do not enhance the effects of rapamycin, further alleviates the concern that AKT suppression may be required in this setting. Nevertheless, these studies suggest that successful agents must promote sustained inhibition of both ERK and mTORC1. Importantly, suppression of these same targets results in tumor regression in a mouse model of NF1-mutant melanoma, underscoring the importance of these pathways in NF1-deficient cancers (4). Nevertheless, establishing the sufficient degree/length of inhibition of both targets that will be required to mediate an efficacious response in patients represents a formidable challenge.

Although mouse models are useful for identifying critical therapeutic targets in genetically defined cancers, the ultimate success of a therapy in humans depends on many factors. Certainly, species-specific differences in tumor complexity may limit efficacy or restrict therapeutic responses to a subset of patients. However, perhaps an even more important consideration relates to dosing. One of the primary obstacles in developing combination therapies, especially when targeting two major signaling pathways, is achieving efficacy while preventing toxicity. As such, even if the correct therapeutic targets have been identified, it may not be possible to sufficiently suppress these targets in humans. MEK inhibitors have been shown to exhibit toxicity in humans at high doses (48, 49). Therefore, in this study, we used a dose of PD-0325901 that is comparable with the tolerable dose in humans. Similarly, the dose of rapamycin was selected based on a previous preclinical study that led to a successful human clinical trial in a number of tuberous sclerosis complex–related pathologies, although reported trough plasma levels were somewhat higher than what has been observed in humans (≤50 ng/mL vs. 3–20 ng/mL in humans; refs. 50–53).

However, our preliminary observations suggest that lower doses and/or intermittent dosing of mTOR inhibitors/rapalogues are also effective when combined with MEK inhibitors. Given the differences in toxicity observed between mice and humans, only clinical trials will reveal whether an effective, nontoxic dose can be achieved. As such, another important goal of this study was to develop a biomarker that could be used to guide dosing in the clinic.

Current clinical trial strategies involve dosing up to the MTD of one drug, and adding the second drug to the tolerable dose when possible. However, it is not always clear how dose escalation/de-escalation affects the degree or kinetics of target inhibition or if dosing at the MTD is necessary. As such, we set out to identify a biomarker that would serve as an early downstream readout of effective, combined inhibition of MEK and TORC1. Although several genes were identified in these tumors, GLUT1 stood out as an important and tractable molecular change. Consistent with the documented role of GLUT1 in regulating glucose uptake, we found that 18F-FDG uptake, as measured by FDG–PET, was a reliable readout of effective, combined target inhibition in vivo. Importantly, changes in GLUT1 expression and 18F-FDG uptake occurred before tumor regression, supporting its role as a molecular marker of TORC1-MEK suppression rather than a consequence of tumor shrinkage. Interestingly, neither GLUT1 expression nor glucose uptake was suppressed after treatment with either rapamycin or MEK inhibitors alone. This observation differs from findings in a subset of other mTOR-driven tumor types, where HIF1α-dependent GLUT1 expression is decreased after treatment with rapamycin, as is 18F-FDG uptake (34, 35). We have previously shown that neither GLUT1 nor HIF1α levels are altered in MPNSTs when treated with rapamycin, marking an important distinction between NF1-deficient tumors and these other mTOR-driven tumors (13). Here, we provide an explanation for this difference, as simultaneous inhibition of both the mTORC1 and MEK is required to suppress GLUT1 and 18F-FDG uptake in MPNSTs. It will be interesting to determine whether inhibition of both pathways is required to alter glucose uptake in other tumors, in particular other RAS-driven tumors. Certainly, other factors, such as hexokinase activity or other members of the GLUT family, may contribute to glucose uptake in some cancers, and in these instances, 18F-FDG uptake might not be an effective biomarker. However, our studies suggest that FDG–PET imaging represents a promising, noninvasive means of measuring combined mTORC1-MEK inhibition in vivo in these NF1-mutant tumors, which can be readily incorporated into clinical trials. Such a tool should help identify the most effective drugs and facilitate dosing, and its utility may extend beyond NF1-mutant cancers.

METHODS

Cell Lines and Reagents

S462 and LN229 cells were purchased from the ATCC. The 90-8TL cells were generously provided by Dr. Eric Legius (KU Leuven, Belgium). The authors performed no further authentication of the cell lines. Cell lines were cultured in DMEM supplemented with FBS (10%) and l-glutamine. Antibodies were obtained from the following sources: Cell Signaling Technologies: pAKT (4060), AKT (9272), pERK (4370), ERK (9102), pS6 (2211), S6 (2217), p110α (4255), p110β (3011), Vinculin (4650), 4E-BP1 (9452), mTOR (7C10), Rictor (53A2), Raptor (24C12), pTSC2 (3611), TSC2 (3612), GAPDH (2118); Santa Cruz Biotechnology: p110α (sc-7176); Trans Labs: p110α (G12920); Sigma: Actin (A2066); Alpha Diagnostics: Glut1 (GT11-A). Torin1, A66-(8), AZD-6284, and CAL-101 were kindly provided by Nathannael Gray (Dana-Farber Cancer Institute/Harvard Medical School). MK-2206 was generously provided by D. Wade Clapp (Indiana University, Indianapolis, IN). GDC-0941 was provided by Genentech. PD-0325901 was a gift from Kevin Shannon (University of California, San Francisco, CA). Rapamycin was purchased from LC Laboratories.

RNAi

Nontargeting and PIK3CA, PIK3CB, PIK3CD, RAPTOR, RICTOR, and mTOR siRNA pools were purchased from Dharmacon (D-001810-10, L-003018-00, L-003019-00, L-006775-00, L-004107-00, L-016984-00, L-003008-00, respectively). siRNAs were transfected overnight in antibiotic-free medium using RNAiMax lipofectamine from Invitrogen.
Cellular Proliferation Studies

Approximately 125,000 cells per well were seeded in 6-well plates. For siRNA experiments, cells were seeded 12 to 16 hours after transfection. Twenty-four hours after plating, day 0 counts were taken using a hemocytometer and trypan blue exclusion. For inhibitor experiments, drug treatments were started at this time. Inhibitors were changed once daily, except in P3K isoform experiments, where drugs were replenished twice daily. Final cell counts were taken 96 hours after day 0 counts.

Drug Treatments and Dosing Schedule

Animal procedures were approved by the Center for Animal and Comparative Medicine in Harvard Medical School in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. CS6/BL6 NPtis mice have been previously described (30). Mice were treated daily with rapamycin via i.p. injections at 5 mg/kg, which were prepared as previously described (13). PD-0325901 was administered at 1.5 mg/kg once or twice daily (10 hours apart) by oral gavage. PD-0325901 was prepared as previously described (54). GDC-0941 was administered at 150 mg/kg once daily by oral gavage. GDC-0941 was prepared as previously described (55). Compounds given in combination were administered sequentially.

Biopsy

Tumor biopsy was performed on mice before drug treatment using the wedge biopsy technique and snap-frozen. Drug treatment was started 8 hours after initial biopsy. The posttreatment biopsy was performed 3 days after treatment began. The mouse was anesthetized by isoflurane inhalation and given a local block with lidocaine and marcaine while the tumor biopsy was collected.

Tumor Volume Measurements

Mice were started on a treatment when tumor size reached 200 to 1,000 mm3. Tumor size was measured every 2 to 3 days by Vernier calipers. Tumor volume was calculated using the standard formula \( V = \frac{4}{3} \pi r^3 \times 0.52 \). A mouse pathologist confirmed that all tumors in this study are MPNSTs.

18F-FDG–PET Imaging and Analysis

PET/CT scans were performed on the Bioscan NanoPET/CT at the Longwood Small Animal Imaging Facility. This PET scanner is equipped with a dedicated isoflurane anesthesia system, temperature-controlled platform, cardiac gating, and respiratory gating. PET scanning was performed on anesthetized animals lying motionless on a table, after retro-orbital i.v. injection of 0.1 to 10 mCi of \(^{18}\)F-FDG PET radioisotope, while being imaged with a coincidence camera. The mice were imaged after a predetermined “washout” period (30–60 minutes). Individual mice were first scanned before treatment and then 40 hours after the treatment regimen was initiated (see dosing schedule methods). For quantitative analysis, the SUV normalized to body weight in the tumor was calculated using SUV = ACvoi (kBq/mL)/FDGdose (MBq)/BW (kg), where ACvoi is the average activity concentration in the tumor volume (or the maximum value), FDGdose is the dose of \(^{18}\)F-FDG administered, and BW is the body weight. For evaluating tumors, the highest SUV in the tumor was taken as the SUVmax.

Microarray

RNA isolated from MPNST tumor samples from NPtis mice treated for 14 hours with vehicle, rapamycin, PD-0325901, or the combination of rapamycin and PD-0325901. For all PD-0325901–treated samples, PD-0325901 was dosed a second time at 10 hours. RNA was isolated with Trizol following the manufacturer’s protocol. RNA clean-up was then performed using the Qiagen RNaseasy Kit (#74104). The Partners HealthCare Center for Personalized Medicine core facility hybridized the RNA to the Affymetrix Mouse Gene 1.0 STS. To determine genes differentially expressed in the combination treatment, a class comparison between the combination-treated samples and all other samples was performed. Analysis was completed using BRB-Array tools developed by Dr. Richard Simon (National Cancer Institute, NIH, Rockville, MD) and the BRB-ArrayTools Development team. Thresholds were set at \( P < 0.001 \). Microarray data can be accessed in the GEO database (accession number GSE57141).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C.F. Malone, J.A. Fromm, T. DeRaedt, K. Cichowski
Development of methodology: C.F. Malone, T. DeRaedt
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.F. Malone, J.A. Fromm, O. Maertens, T. DeRaedt, R. Ingraham, K. Cichowski
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.F. Malone, J.A. Fromm, O. Maertens, T. DeRaedt, K. Cichowski
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Study supervision: K. Cichowski

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REFERENCES


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